

Optical Properties of Viruses

James Vaughn
Dept. of Microbiology
11 Hills Beach Rd.
Biddeford, ME 04005
Phone: (207)283-0170 Fax: (207)283-3249 email: jvaughn@mailbox.une.edu

William M. Balch
Bigelow Laboratory for Ocean Sciences
P.O. Box 475, McKown Point
W. Boothbay Harbor, ME 04575
Phone: (207) 633-9600 Fax: (207) 633-9641 e-mail: bbalch@bigelow.org

James Novotny
Dept. of Microbiology
11 Hills Beach Rd.
Biddeford, ME 04005
Phone (207)283-0170 Fax: (207)283-3249 e-mail: jnovotny@mailbox.une.edu

AWARD #: N00014-96-1-0999

LONG-TERM GOALS

The long-range goal of this ONR-sponsored research is to document the importance of viruses to light scattering in the sea.

OBJECTIVES

The objectives of this work are to: 1) understand the optical properties of some common viruses, 2) examine the optical impact of several common marine bacteriophages, and 3) assess how important viruses are to the total absorption, scattering and backscattering of dissolved organic matter in sea water. Such results will be directly applicable to bio-optical models.

APPROACH

The first year of this project focused on understanding the optical properties of some common viruses. The rationale behind beginning with these types of viruses is that they were easily propagated, they could be grown in large concentrations, and they were simple to enumerate. Viruses were propagated on bacterial host cultures. Size distribution of the viral suspensions were quantified using flow-field fractionation. A Wyatt Technologies Light scattering photometer was used to estimate the volume scattering of these particles.

The second year of this project extended our experiments from common bacteriophage of *E. coli*, to marine bacteriophage. This required isolation and propagation of the host bacteria, then screening the host for viral infectants, and subsequent propagation and concentration of the viruses. We also performed experiments in which viral infection of host bacteria was followed two ways: 1) using traditional plaque formation techniques, and 2) using optical measurements.

Report Documentation Page				Form Approved OMB No. 0704-0188	
Public reporting burden for the collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to a penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.					
1. REPORT DATE 1998		2. REPORT TYPE		3. DATES COVERED 00-00-1998 to 00-00-1998	
4. TITLE AND SUBTITLE Optical Properties of Viruses				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S)				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of New England, Department of Microbiology, 11 Hills Beach Rd, Biddeford, ME, 04005				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release; distribution unlimited					
13. SUPPLEMENTARY NOTES See also ADM002252.					
14. ABSTRACT					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Same as Report (SAR)	18. NUMBER OF PAGES 5	19a. NAME OF RESPONSIBLE PERSON
a. REPORT unclassified	b. ABSTRACT unclassified	c. THIS PAGE unclassified			

WORK COMPLETED

Virus Propagation, Purification and Enumeration

Bacterial viruses and their appropriate hosts were obtained from the American Type Culture Collection (ATCC, Bethesda, MD). Following the first year experiments, some follow-up experiments were performed with MS-2, ATCC 15597 B-1 (host cell, *E. coli* ATCC #15597), which is a small icosahedral virus (25-30nm) containing single-stranded RNA. We also repeated some experiments using T-4, ATCC 11303 B-1 (host cell, *E. coli* ATCC #11303), a 225 nm double-stranded DNA phage possessing an elongated head and tail structure. Following these experiments, we isolated two strains of marine virus from waters off of UNE and Bigelow Laboratory. One strain, Y1, had a yellow bacterial host (as yet unidentified), another viral strain isolated off the BLOS dock was C-1, with a colorless bacterial host. Bacterial host stocks were cultured in tryptone broth. Short term storage of host cultures involved the preparation of cultures on tryptone agar slants. Following inoculation, cultures were incubated (36 C) overnight. Slants could be stored at 4°C for up to two weeks. For long term storage, overnight broth cultures were supplemented with 10% sterile glycerol, dispensed into 1 ml aliquots in cryovials, and stored frozen at -74°C. Bacteriophage MS-2 and T-4 stocks were prepared in a similar manner. Overnight cultures of respective host cells were inoculated into tryptone broth and incubated with shaking for 2 hr. One-ml volumes of appropriate phage stock were then added, and the incubation continued for an additional 4 hr. Harvested lysates were centrifuged (5000xg/15 min) to remove debris, and further clarified via filtration (0.45 um). Resulting bacteriophage titers, enumerated by a plaque assay method (Adams 1959), averaged 10^{11} pfu/ml for MS-2, 10^{10} pfu/ml for T-4, 10^{11} pfu/ml for Y-1, and # pfu/ml for C-1. Phage stocks were stored in 1 ml aliquots at -74°C.

High titer, purified, phage stocks used in optical measurement experiments were prepared in the following manner. Overnight host cell cultures were inoculated into 1 L volumes of tryptone broth and incubated with shaking for 2 hr. Cultures were then inoculated with 10^{10} to 10^{11} pfu of appropriate bacteriophage and incubation continued for 4 hr. Lysates were then centrifuged and filtered as described above, and the phage concentrated by ultracentrifugation (230,000xg/2 hr). Resulting pellets were resuspended in 10 ml of phosphate buffered saline (PBS). Concentrates were layered onto cesium chloride gradients (1.3 - 1.7 g/ml) and centrifuged (100,000 xg/60 min/10C), as per Bachrach and Freidman (1971). Resulting virus bands were collected and dialyzed overnight (10°C) against 1/2 strength PBS. Purified, concentrated virus stocks were assayed and stored at 4°C until used. Stocks were usually prepared several days prior to each experiment. None were stored for more than 5 days before use. Phage concentrations in purified stocks averaged 10^{12} - 10^{14} pfu/ml.

Sybr-Green Viral Enumeration

In order to check the accuracy of the backscattering cross-section estimates, the Sybr-Green technique of Noble and Fuhrman (1998) was implemented for comparison with the standard plaque assay techniques used in years 1 and 2. The technique essentially involves filtering virus particles onto a 20nm pore-size filter, labeling virus particles with a fluorescent dye, rinsing unbound dye off the particles, and counting the fluorescently labelled viruses at 1000X under epifluorescent illumination.

Size Distribution Measurements

One needs to know the optical as well as physical cross-sections of the particles for calculation of absorption, scattering and backscattering efficiencies. Due to the small size of viral particles, Coulter counting cannot be effectively used to estimate the size spectrum of a viral suspension. The most effective means to acquire such a size spectrum is with field-flow fractionation (FFF). This technique was used in year 1, and in year 2, we also performed parallel transmission electron microscopy (TEM) to verify the size. FFF is a chromatographic-like separation which occurs in a thin ribbon-shaped channel as an external field and/or pressure gradient is applied perpendicularly to the flow channel. Typically used fields or gradients for fractionation are sedimentation, thermal or cross-flow; FFF allows fractionation of materials from a few thousand molecular weight to 100mm particle diameters. FFF has been used previously to size fractionate viruses (Giddings et al, 1980). In the context of these experiments, FFF allows identification of viral aggregates and membrane fragments from solitary viruses for subsequent optical measurements.

Optical Measurements

In all experiments, a Dawn Laser Light Scattering Photometer was used to measure the volume scattering function, and to calculate the backscattering coefficient (b_b). This instrument makes 400 measurements of the phase function each second and averages the data over any pre-set time period. The Wyatt Technologies Dawn Photometer is equipped with argon ion laser light source (512 nm) with capability for discrete or continuous flow-through measurements.

Effects of Virus Concentration on Optical Properties

Following each optical measurement, the viral cultures were serially diluted and re-assayed to verify the concentration dependence of viruses on their associated optical properties. Observations also were made to determine the impact of phage on the optical properties of the host bacteria, before and after viral attachment. Initial experiments with MS-2 and T-4 were conducted in phosphate buffered saline (pH 7.2). Once absorption, and scattering characteristics were established, serial dilution experiments were repeated in ultra-filtered sea water from the Gulf of Maine into which a known titer of the above viruses was placed. This last step, while not the same as adding marine viruses to sea water, provided a preliminary examination of viral optical properties in sea water. The same protocol was followed for the two new marine strains, Y-1 and C-2 and compared with the original E. coli bacteriophage, MS-2 and T-4.

RESULTS

Our results have quantified the backscattering cross section for the four above-named viruses. Serial dilution experiments demonstrated a viral impact on backscattering at the upper-end of the concentration range found in seawater, in addition, the linearity of backscattering versus dilution experiments was strong. A medical application of this work is that we now have a quick means to optically measure viral titer of a solution. The shape of the volume scattering function was quite different for the four viral types, although one clear consistency is the relative flatness of the VSF. It should also be noted that the backscattering cross sections were similar to published values which were based on Mie scattering calculations (Stramski and Mobley, 1997). Moreover, viral aggregates were not an issue given that flow field fractionation demonstrated viral particles to be mostly solitary (i.e. the

particle spectrum for our viral suspensions shows a mode at the diameter of the viruses based on electron micrographs).

To insure that viral abundances (used in calculating scattering cross-sections) are accurate, we have employed the Sybr-Green technique (Nobel and Fuhrman, 1998). We have run parallel experiments in which traditional plaque assay counts were compared to the Sybr-Green counts. Results show that the viral counts are identical with the two techniques.

Infection experiments were performed with two of our virus strains, MS-2 and T-4. The experiments involved uninfected controls for comparison. Within one or two infection cycles, most of the particulate backscattering was converted to Rayleigh scattering. In short, turbid bacterial cultures were clarified in ~90 minutes, relative to the uninfected control.

IMPACT/APPLICATIONS

Results from all of our dilution experiments (year 1 and 2) are useful for understanding the overall role of viruses in the sea, and answering, once and for all, the suggestion of Morel and Ahn (1991) who postulated that viruses might represent a significant fraction of the "missing" backscattering in the sea. At present, we would concur with others that viruses, while a significant source of backscattering, are not the most important source of backscattering in the sea. Agreement of plaque assay and Sybr-green counts provides additional confidence that the backscattering cross-section estimates are accurate.

While viruses individually do not account for a large fraction of the "missing backscattering" in the sea, preliminary infection experiments show profound effect of viruses in converting particulate backscatterers to Rayleigh scatterers, at an impressive rate. Due to the rapid infection and propagation stages of viruses, the clarification of a bacterial culture by the host-specific virus takes several hours, extraordinarily rapid as compared to other bio-optical processes in the sea.

TRANSITIONS

Given the successful, reproduceable, dilution experiments on four bacteriophage strains (2 freshwater, and 2 marine), as well as infection experiments on MS-2 and T-4 viruses, attention is being directed to infection experiments using the marine bacteriophages Y-1 and C-2.

RELATED PROJECTS

This DEPSCoR work is a joint venture between Bigelow Laboratory and their affiliate, the University of New England. Collaborative relationships are maintained with Dr. Ken Voss and Dr. Howard Gordon, both ONR-funded investigators at the University of Miami Dept. of Physics.

REFERENCES

Adams, M. H. 1959. Bacteriophages. Interscience. New York, NY.

Bachrach, U. And A. Freidman. 1971. Practical procedures for the purification of bacterial viruses. Appl. Microbiol. 22: 706-715.

Giddings, J. C., M. N. Myers, K. D. Caldwell, and S. R. Fisher. 1980. Methods of Biochemical Analysis. Vol. 26, D. Glick ed. John Wiley, New York. pp. 79-136.

Morel, A. and Y. Ahn. 1991. Optics of heterotrophic nanoflagellates and ciliates: A tentative assessment of their scattering role in oceanic waters compared to those of bacterial and algal cells. *Journal of Marine Research*, 49, 177-202.

Noble, R. T. and J. A. Fuhrman. 1998. Use of SYBR Green I for rapid epifluorescence counts of marine viruses and bacteria. *Aquatic Microbial Ecology* 14: 113-118.

Stramski, D. and C. D. Mobley. 1997. Effects of microbial particles on oceanic optics: A database of single-particle optical properties. *Limnol. Oceanogr.* 42: 538-549.